Acid regulation of NaDC-1 requires a functional endothelin B receptor

Liping Liu[1,7], Miriam Zacchia[2,7], Xuefei Tian[2,7], Laxiang Wan[2], Aiji Sakamoto[3], Masashi Yanagisawa[4,5], Robert J. Alpern[1,2] and Patricia A. Preisig[1,2,6]

[1]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA; [2]Department of Medicine, Yale University, New Haven, Connecticut, USA; [3]Department of Bioscience, Division of Biotechnology, National Cardiovascular Center Research Institute, Osaka, Japan; [4]Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas, USA; [5]Department of Genetics, University of Texas Southwestern Medical Center, Dallas, Texas, USA and [6]Department of Cellular and Molecular Physiology, Yale University, New Haven, Connecticut, USA

Metabolically generated acid is the major physiological stimulus for increasing proximal tubule citrate reabsorption, which leads to a decrease in citrate excretion. The activity of the Na-citrate cotransporter, NaDC-1, is increased *in vivo* by acid ingestion and *in vitro* by an acidic pH medium. In opossum kidney cells the acid stimulatory effect and the ability of endothelin-1 (ET-1) to stimulate NaDC-1 activity are both blocked by the endothelin B (ET_B) receptor antagonist, BQ788. Acid feeding had no effect on brush border membrane NaDC-1 activity in mice in which ET_B receptor expression was knocked out, whereas a stimulatory effect was found in wild-type mice. Using ET_A/ET_B chimeric and ET_B C-terminal tail truncated constructs, ET-1 stimulation of NaDC-1 required a receptor C-terminal tail from either ET_A or ET_B. The ET-1 effect was greatest when either the ET_B transmembrane domain and C-terminal tail were present or the ET_B C-terminal tail was linked to the ET_A transmembrane domain. This effect was smaller when the ET_B transmembrane domain was linked to the ET_A C-terminal tail. Thus, the acid-activated pathway mediating stimulation of NaDC-1 activity requires a functional ET_B receptor *in vivo* and *in vitro*, as does acid stimulation of NHE3 activity. Since increased NaDC-1 and NHE3 activities constitute part of the proximal tubule adaptation to an acid load, these studies indicate that there are similarities in the signaling pathway mediating these responses.

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Correspondence: *Patricia A. Preisig, Department of Internal Medicine, Section of Nephrology, Yale University, 300 Cedar Street, TAC S363, P.O. Box 208029, New Haven, Connecticut 06520-8029, USA. E-mail: Patricia.Preisig@Yale.edu*

[7]These authors contributed equally to this study.

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Diet-associated acid loads are the major determinant of urinary citrate excretion. Urinary citrate is an important inhibitor of nephrolithiasis and nephrocalcinosis, as it complexes urinary calcium, preventing the calcium from forming insoluble complexes with oxalate and other anions. Hypocitraturia is present in about half of recurrent stone forming patients and its presence correlates highly with the dietary acid load.[1,2] Chronic metabolic acidosis, chronic potassium deficiency (a model of low intracellular pH), and diets high in animal protein reduce urinary citrate excretion and significantly increase the risk for kidney stone formation.[2–10] Understanding the mechanism by which dietary acid reduces citrate excretion is essential to identifying mechanisms by which the risk for kidney stone formation can be reduced.

Citrate is freely filtered at the glomerulus and the magnitude of citrate excretion is determined solely by its rate of reabsorption and metabolism in the kidney proximal tubule. Citrate is transported across both the apical and basolateral membranes by Na-coupled, electrogenic transporters. The Na-dependent dicarboxylate transporter isoform-1 (NaDC-1) mediates citrate uptake across the apical membrane, and is stimulated by acidic intracellular pH and dietary acid loads.[11–16] Reabsorbed citrate is metabolized in the proximal tubule. NaDC-3 mediates citrate uptake across the basolateral membrane.[14,16,17] However, unlike transport across the apical membrane, citrate transport across the basolateral membrane is inhibited by acidic pH. The rate and magnitude of reabsorption across the apical membrane is the major determinant of citrate excretion, and, thus, the risk for kidney stone formation.

To elucidate the acid-activated pathway that mediates acid stimulation of NaDC-1 activity we used an opossum kidney cell line (OKP) that possesses many characteristics of the renal proximal tubule.[18] The OKP Na-dependent dicarboxylate cotransporter (oNaDC-1) expressed in *Xenopus* oocytes shows functional characteristics similar to those of the rat, rabbit, and human apical membrane NaDC-1 protein.[19] These studies conducted in both OKP cells and mice

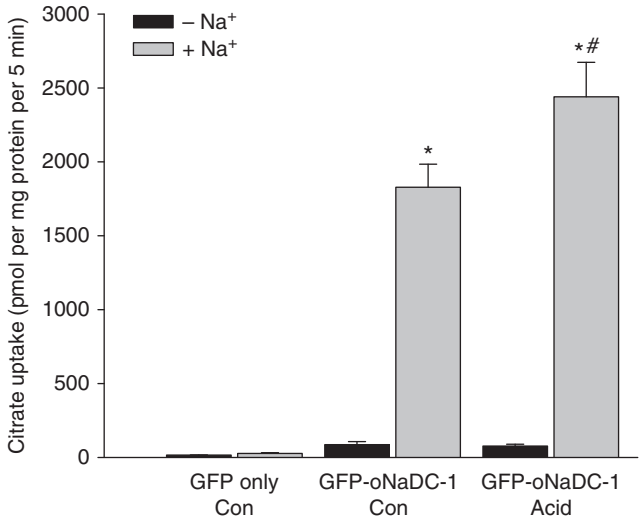

Figure 1 | Opossum kidney cell line (OKP) cells transfected with OKP Na-dependent dicarboxylate cotransporter-1 (oNaDC-1) show acid-regulated Na-dependent citrate uptake. OKP cells were exposed to control (pH 7.4) or acid (pH 6.8) media × 6 h. oNaDC-1 activity was assayed using [^{14}C]-citrate uptake and expressed as pmol of citrate/mg protein/5 min. *$P<0.05$ versus minus Na^+; #$P<0.05$ versus plus Na^+ control. $n=3$ for green fluorescent protein (GFP) only; $n=12$ for GFP-oNaDC-1 Con (control) and acid groups.

demonstrate that media acidification or acid feeding, respectively, requires a functional endothelin B (ET_B) receptor to stimulate NaDC-1 activity.

RESULTS

OKP cells transfected with oNaDC-1 shows acid regulation of transporter activity

Native OKP cells show minimal [^{14}C]-citrate uptake in the absence or presence of Na, in spite of the fact that they express oNaDC-1 mRNA. Therefore, we overexpressed oNaDC-1 in all studies. OKP cells transfected with the oNaDC-1 construct (green fluorescent protein (GFP)-oNaDC-1) show Na-dependent [^{14}C]-citrate uptake at rates that are significantly higher than observed in cells transfected with GFP only (Figure 1). Media acidification has no effect on [^{14}C]-citrate uptake in the absence of Na, but significantly increases the rate of citrate uptake in the presence of Na (Figure 1).

oNaDC-1 is stimulated by media acidification in a dose- and time-dependent manner

OKP cells transfected with oNaDC-1 and exposed to media of pH 7.4, 7.3, 7.2, 7.0, or 6.8 show pH-sensitive [^{14}C]-citrate uptake, with the highest rate of uptake at pH 6.8 (Figure 2a). Activation with pH 6.8 media begins within 2 h and persists for 24 h (Figure 2b).

oNaDC-1 is stimulated by ET-1 in a dose- and time-dependent manner in the presence of the ET_B receptor

OKP cells transfected with oNaDC-1 and ET_B, and then exposed to vehicle (acetic acid) or ET-1 (10^{-10} to 10^{-7} mol/l)

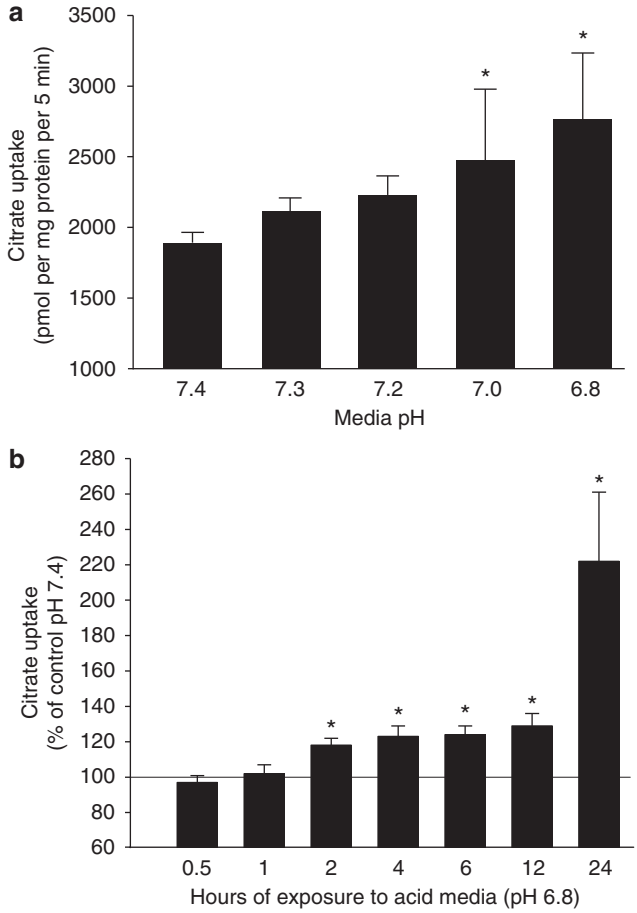

Figure 2 | Opossum kidney cell line (OKP) cells transfected with OKP Na-dependent dicarboxylate cotransporter-1 (oNaDC-1) show pH- and time-dependent increases in citrate uptake after media acidification. (**a**) pH is noted on the *x*-axis; *y*-axis: citrate uptake expressed as pmol/mg protein/5 min. *$P<0.05$ versus pH 7.4. $n=9$ for each pH. (**b**) *x*-axis: length of exposure to acidic media; *y*-axis: acid:control ratio at each time point. *$P<0.05$ versus no percentage change in the ratio (100). $n=9$ for all time points except 12 h, where $n=8$. oNaDC-1 activity was assayed using [^{14}C]-citrate uptake.

show dose-dependent stimulation of [^{14}C]-citrate uptake with a significant effect observed at ET-1 concentrations of 10^{-8} mol/l or higher (Figure 3a). Significant activation with 10^{-8} mol/l ET-1 begins within 15 min and lasts for 4 h (Figure 3b).

oNaDC-1 is stimulated by acid and ET-1 signaling through the ET_B, but not the ET_A receptor

We have previously shown that acid stimulation of another proximal tubule brush border membrane transporter, NHE3, is mediated by autocrine activation of the ET_B receptor by ET-1.[20] To determine the receptor requirement for ET-1 stimulation of oNaDC-1 activity, studies were conducted in the presence or absence of transfected ET_B receptor and in the presence or absence of a specific ET_B receptor blocker, BQ788.[21] As shown in Figure 4a, cells transfected with the ET_B receptor show greater citrate uptake when

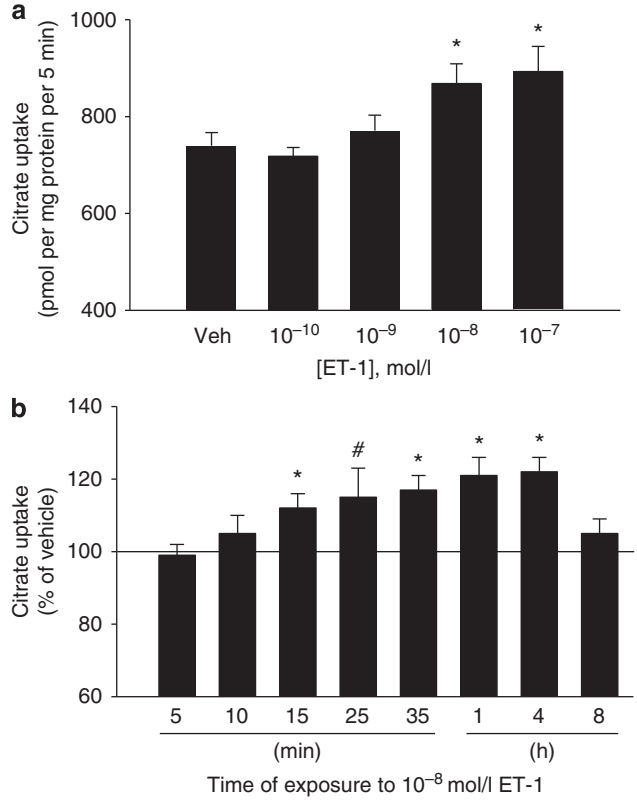

Figure 3 | Opossum kidney cell line (OKP) cells transfected with OKP Na-dependent dicarboxylate cotransporter-1 (oNaDC-1) and endothelin B $(ET)_B$ show both dose- and time-dependent ET-1 stimulation of oNaDC-1 activity. (**a**) Dose-dependent study. oNaDC-1 activity was assayed as [^{14}C]-citrate uptake after 35 min exposure to the indicated ET-1 concentrations. *$P<0.05$ versus vehicle. $n=9$ for all ET-1 concentrations. (**b**) Time-dependent study after exposure to 10^{-8} mol/l ET-1. *$P<0.05$ versus vehicle; $^{\#}P=0.09$ versus vehicle. $n=12$ for studies at 5, 10, 15, and 25 min; $n=9$ for studies at 35 min and 1 h; $n=6$ for studies at 4 and 8 h.

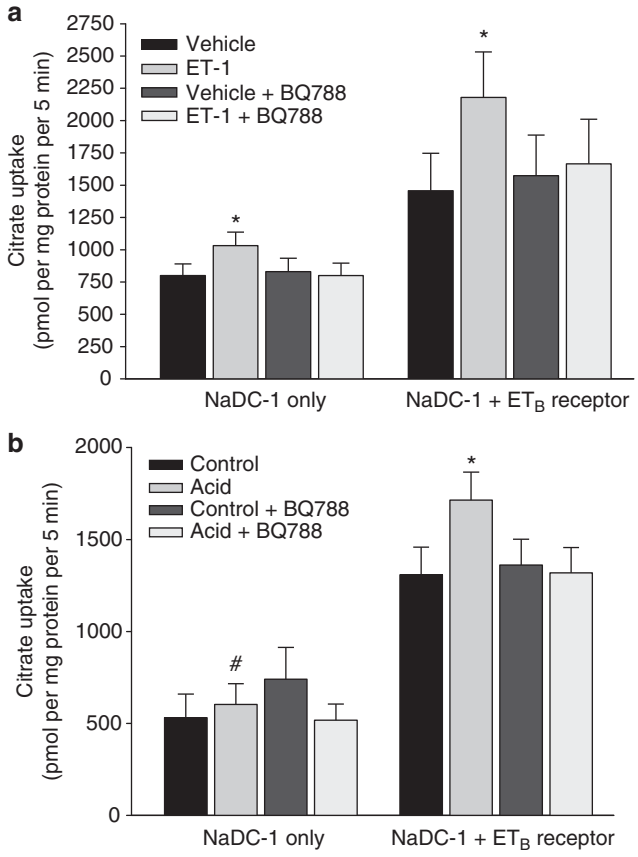

Figure 4 | Endothelin-1 (ET-1) stimulation of opossum kidney cell line Na-dependent dicarboxylate cotransporter-1 (oNaDC)-1 activity is dependent on the ET_B receptor. Cells were co-transfected with oNaDC-1 alone (left set of bars) or oNaDC-1 + ET_B (right set of bars). oNaDC-1 activity was assayed as [^{14}C]-citrate uptake and reported as pmol/mg protein/5 min. (**a**) Cells were exposed to vehicle or 10^{-8} mol/m ET-1 × 35 min. $n=8$ for vehicle and $n=9$ for BQ788 studies. *$P<0.01$ versus vehicle. (**b**) Cells were exposed to media of pH 7.4 (con) or 6.8 (acid) × 6 h. $n=8$ for all studies. *$P<0.05$ versus pH 7.4; $^{\#}P=0.06$ versus pH 7.4.

exposed to 10^{-8} mol/l ET-1. The ET_B receptor blocker, BQ788, blocks entirely ET-1 stimulation of [^{14}C]-citrate uptake in cells transfected with vector alone (native ET_B receptor only) or the ET_B receptor (native and exogenous ET_B receptor).

Similarly, the effect of media acidification on [^{14}C]-citrate uptake is greater in cells transfected with the ET_B receptor (native and exogenous ET_B receptor) compared with cells transfected with vector alone (native ET_B receptor only), and BQ788 blocks the effect of media acidification in both situations (Figure 4b). Thus, both acid and ET-1 stimulation of oNaDC-1 activity requires the ET_B receptor.

In contrast, as shown in Figure 5 (right set of bars), when cells are transfected with the ET_A receptor, 10^{-8} mol/l ET-1 decreases, rather than increases [^{14}C]-citrate uptake. In addition, comparing the left set of bars (cells transfected with NaDC-1 only, native ET_B only) with the right set of bars (cells transfected with NaDC-1 + ET_A, native ET_B present), overexpressing the ET_A receptor inhibits baseline activity. A possible explanation for this observation is that in the absence of ligand (right set of bars, vehicle (first bar) and vehicle + BQ788 (third bar)) the 'empty' ET_A receptor inhibits baseline oNaDC-1 activity (compare bars 1 and 3 in the right set to bars 1 and 3 in the left set), and ligand (ET-1) binding to the overexpressed ET_A receptor (right set of bars, bar 2) causes an ET-1 induced inhibition of oNaDC-1 activity, whereas ligand binding to the ET_B receptor in the absence of ET_A receptor expression (left set of bars, bar 2) stimulates oNaDC-1 activity. Thus, in the presence of transfected ET_A and endogenous ET_B receptor (right set of bars), the effect of the ET_A receptor predominates, and baseline and ET-1 regulated oNaDC-1 activities decrease. This hypothesis predicts that in ET_A-transfected cells exposed to BQ788 (no functional ET_B receptor present), ET-1 should again inhibit oNaDC-1 activity. As shown in Figure 5 (right set of bars, fourth bar), oNaDC-1 activity is lower than baseline (compared with the first bar in the set), but did not achieve statistical significance.

Location of oNaDC-1 expression associated with acid and ET-1 stimulation of oNaDC-1 activity

To determine whether exposure to media acidification (Figure 6a) or ET-1 (Figure 6b) affects the intracellular location of transfected oNaDC-1, confocal microscopy studies were carried out making use of the GFP tag on oNaDC-1. In control-transfected cells, although the majority of GFP-oNaDC-1 expression is located in the cytoplasm, there is expression on the apical side of the cell, but no detectable expression on the basolateral side. The apical expression likely accounts for the increased citrate uptake in transfected control cells compared with untransfected cells (Figure 1). After exposure to either acid pH or ET-1, GFP-oNaDC-1 expression moves from a predominantly cytoplasmic location to the apical side of the cell, still with no detectable expression on the basolateral side. Thus, although post-translational modification of apically expressed NaDC-1 cannot be ruled out, the parallel increase in expression on the apical side of the cell and NaDC-1 activity strongly suggests that trafficking from a cytoplasmic pool to the apical membrane contributes significantly to the increase in NaDC-1 activity.

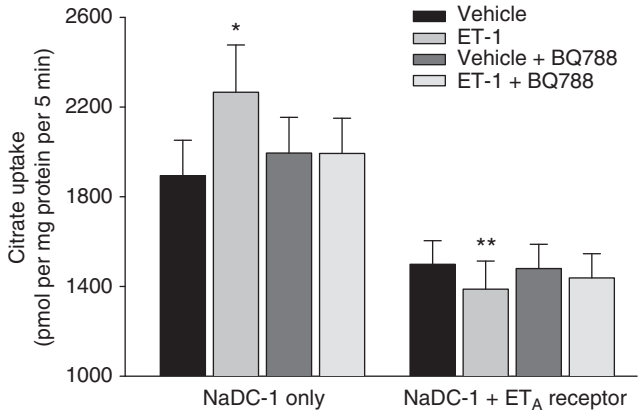

Figure 5 | Endothelin-1 (ET-1) does not stimulate opossum kidney cell line Na-dependent dicarboxylate cotransporter-1 (oNaDC-1) activity in opossum kidney cells transfected with ET_A. Cells were cotransfected with oNaDC-1 or oNaDC-1 + ET_A. oNaDC-1 activity was assayed as [^{14}C]-citrate uptake. *$P<0.001$ versus vehicle; **$P<0.01$ versus vehicle $n=9$ for all studies.

Acid stimulation of oNaDC-1 activity is mediated by the ET_B receptor *in vivo*

To confirm that ET-1/ET_B signaling is required for acid stimulation of NaDC-1 activity *in vivo*, studies were carried out in wild-type ($ET_B^{+/+}$) and ET_B receptor-deficient ($ET_B^{-/-}$) mice. The $ET_B^{-/-}$ mice were created by removing exon 3 of the coding sequence and replacing it with a neo-cassette.[22] The phenotype, which is neonatally lethal, was rescued by expression of a *dopamine β-hydroxylase* promoter/ET_B receptor transgene.[22–24] In the rescued mice, confirmation that the ET_B receptor was not expressed in the kidney was obtained from polymerase chain reaction studies carried out using a forward primer from exon 1 and a reverse primer from exon 3. The results showed a polymerase chain reaction product of ~687 bp in renal cortex harvested from $ET_B^{+/+}$ animals, but no polymerase chain reaction product in cortex harvested from $ET_B^{-/-}$ animals (data not shown).

Mice had *ad lib* access to either regular drinking water (control) or drinking water containing 0.3 mol/l NH_4Cl

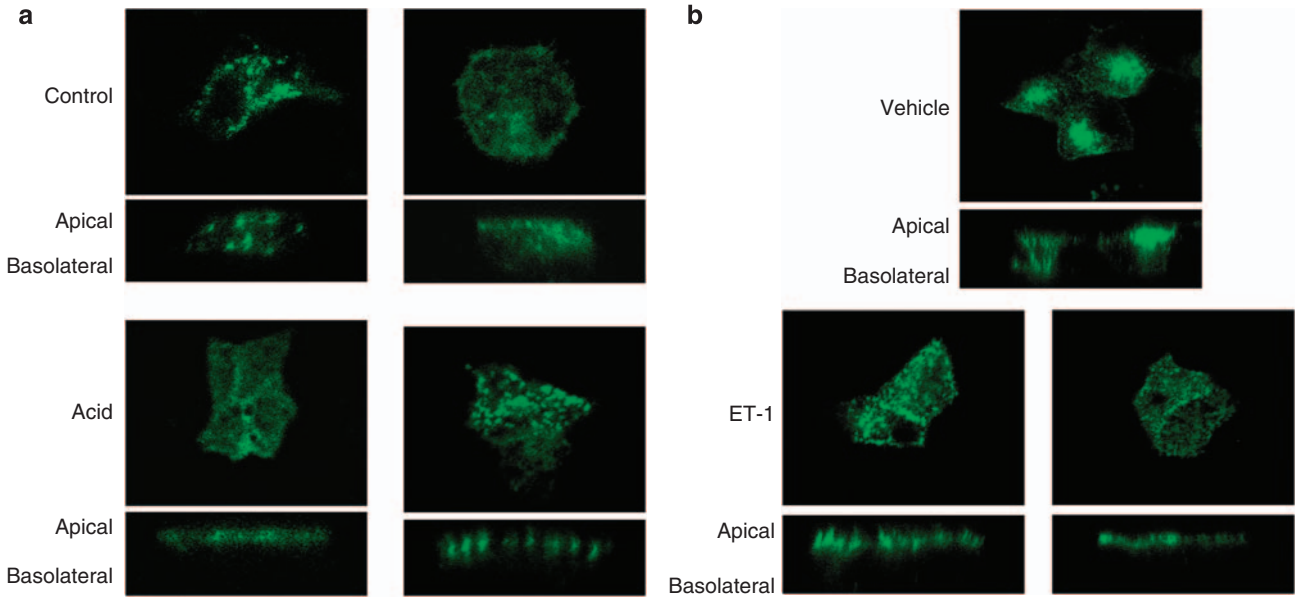

Figure 6 | Apical membrane opossum kidney cell line Na-dependent dicarboxylate cotransporter-1 expression increases after either media acidification or exposure to 10^{-8} mol/l endothelin-1 (ET-1). Cells were prepared for confocal microscopy as described in the Methods section. (**a**) Green fluorescent protein (GFP) expression comparing exposure to control (pH 7.4) and acid (pH 6.8) media × 6 h. (**b**) GFP expression comparing exposure to vehicle and 10^{-8} mol/l ET-1 × 35 min. Typical pictures for both panels.

Table 1 | Arterial blood gases and plasma chemistries

	Endothelin$_B^{+/+}$ mice		Endothelin$_B^{-/-}$ mice	
	Control, *n*=16–18	Acid, *n*=16–18	Control, *n*=16–18	Acid, *n*=16–18
pH	7.23 ± 0.01	7.09 ± 0.02*	7.25 ± 0.01	7.11 ± 0.02*
pCO_2, mm Hg	52 ± 1	55 ± 2	51 ± 2	56 ± 3
[HCO_3^-], mM	21.7 ± 0.3	16.6 ± 0.5*	22.2 ± 0.4	17.7 ± 0.7*
Δ[HCO_3^-], mM	−5.06 ± 0.60		−4.43 ± 0.93	
Na^+, mmol/l	144 ± 1	148 ± 1*	143 ± 1	150 ± 1*
K^+, mmol/l	3.8 ± 0.1	4.3 ± 0.2	4.1 ± 0.1	4.5 ± 0.1
Cl^-, mmol/l	116 ± 1	123 ± 1*	115 ± 1	124 ± 1*

*$P<0.05$ versus respective control group.

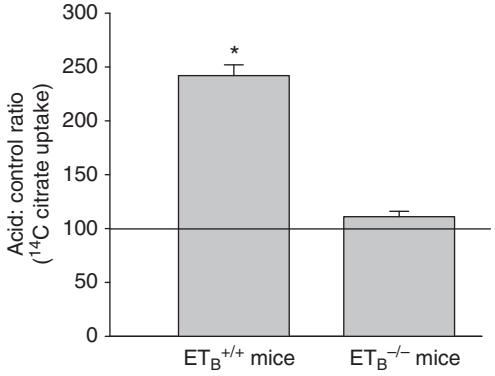

Figure 7 | Stimulation of opossum kidney cell line Na-dependent dicarboxylate cotransporter-1 activity by acid feeding is dependent on the (endothelin B) ET_B receptor. $ET_B^{+/+}$ and $ET_B^{-/-}$ mice were provided with plain drinking water or drinking water containing 0.3 mol/l NH_4Cl *ad lib* × 7 days. Kidney cortex was harvested, brush border membrane vesicles prepared, and Na-dependent [^{14}C]-citrate uptake measured as described in the Methods section. All assays were carried out in triplicate and meaned to obtain a transport rate for this study. *$P<0.05$ versus mice drinking plain water. $n=6$ for all groups.

(acid). As shown in Table 1, after 7 days of acid feeding, blood pH and [HCO_3] are significantly decreased in both acid fed groups compared with their respective control groups, with blood [HCO_3] decreased to a similar extent in the two groups. In addition, there are no differences in arterial blood gases between the $ET_B^{+/+}$ and $ET_B^{-/-}$ mice. As shown in Figure 7, Na-dependent [^{14}C]-citrate uptake, assayed in brush border membrane vesicles (BBMVs) harvested from renal cortex, is significantly increased by acid feeding to $ET_B^{+/+}$ mice, but acid feeding is without effect on Na-dependent [^{14}C]-citrate uptake in the $ET_B^{-/-}$ mice. Although baseline (control) NaDC-1 activity seemed increased in the $ET_B^{-/-}$ mice, it is not significantly different than baseline NaDC-1 activity in the $ET_B^{+/+}$ mice. Thus, in both *in vitro* and *in vivo* models of an acid load the presence of a functional ET_B receptor is required for acid stimulation of NaDC-1 activity.

A C-terminal tail is required for ET-1 stimulation of oNaDC-1 activity

To elucidate the region(s) of the ET_B receptor required for ET-1 stimulation of oNaDC-1 activity, studies were conducted using ET_A/ET_B chimeric and ET_B C-terminal deletion constructs. ET_A and ET_B receptors exhibit 55% overall amino acid homology.[25,26] Within the receptor domains, the C-terminal region has the least amount of homology and the transmembrane (TM) domains the most homology.

As shown in previous studies, OKP cells transfected with the wild-type ET_B receptor showed ET-1-induced stimulation of NaDC-1 activity (Figure 8, first bar), whereas cells transfected with the ET_A receptor showed no stimulation (Figure 8, fourth bar). Removing the C-terminal tail from the ET_B receptor (Figure 8, ET_B no tail, second bar) prevents ET-1 stimulation of oNaDC-1, showing that a C-terminal tail is required. Replacing the ET_B C-terminal tail with the ET_A C-terminal tail (Figure 8, ET_B TM/ET_A tail, third bar) significantly reduces the ET-1 effect (Figure 8, compare first and third bars), but a significant effect is still observed. Replacing the ET_A C-terminal tail with the ET_B C-terminal tail (Figure 8, ET_A TM/ET_B tail, fifth bar) restores ET-1 stimulation of oNaDC-1 activity to a magnitude not different than that observed with the wild-type ET_B receptor (Figure 8, compare first and fifth bars).

There is no difference in the minimal ET-1 effect when comparing cells transfected with ET_B no tail (second bar), ET_B TM/ET_A tail (third bar), and wild-type ET_A (fourth bar) (all comparisons non-significant), and the minimal ET-1 effect observed in these three groups is significantly smaller than that observed when cells are transfected with wild-type ET_B receptor (compare first bar with second–fourth bars). Taken together, these studies show that (1) the ET_B receptor requires a C-terminal tail for ET-1 stimulation of oNaDC-1 activity, (2) the ET-1 effect is greatest when both the ET_B TM and C-terminal regions of the receptor are present or when the ET_B C-terminal domain is linked to the ET_A TM domains, (3) ET-1 does not increase oNaDC-1 activity in the presence of the wild-type ET_A receptor. Thus, maximal ET-1 stimulation requires the presence of the ET_B C-terminal tail.

ET_B C-terminal region required for ET-1 stimulation of oNaDC-1 activity

On the basis of above results, (1) a C-terminal tail must be present and (2) the wild-type ET_B, not the wild-type ET_A receptor is required for ET-1 stimulation of NaDC-1 activity.

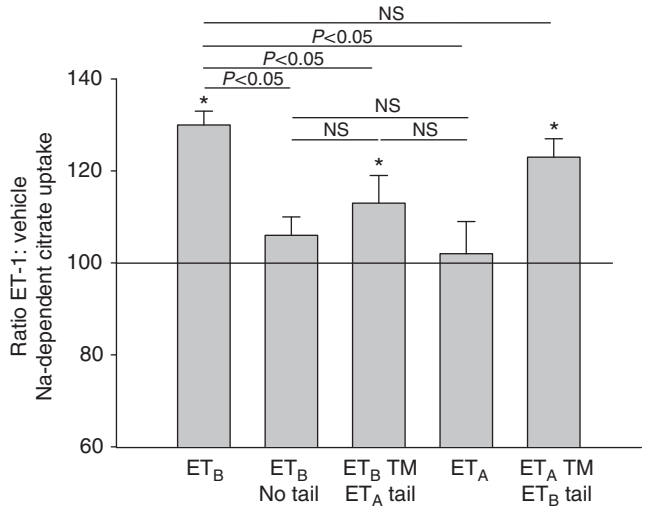

Figure 8 | Endothelin B (ET_B) C-terminal tail required for ET-1 stimulation of opossum kidney cell line Na-dependent dicarboxylate cotransporter-1 (oNaDC-1) activity. Cells were transfected with oNaDC-1 and the wild-type or chimeric receptor as indicated on the *x*-axis and [^{14}C]-citrate uptake was measured. ET_A: wild-type ET_A receptor; ET_B: wild-type ET_B receptor; ET_B no tail: ET_B receptor without a C-terminal tail; ET_B TM/ET_A tail: chimeric receptor including the ET_B transmembrane (TM) domains and ET_A C-terminal tail; ET_A TM/ET_B tail: chimeric receptor including the ET_A transmembrane domains and ET_B C-terminal tail. Statistical results from the one-way analysis of variance are included in the figure. $^*P<0.05$ for the ET-1:vehicle ratio being statistically greater than 100. $n=9$ for ET_A and ET_B no tail studies; $n=18$ for ET_A TM/ET_B tail and ET_B TM/ET_A tail studies; $n=23$ for wild-type ET_B studies. Abbreviation: NS, not significant.

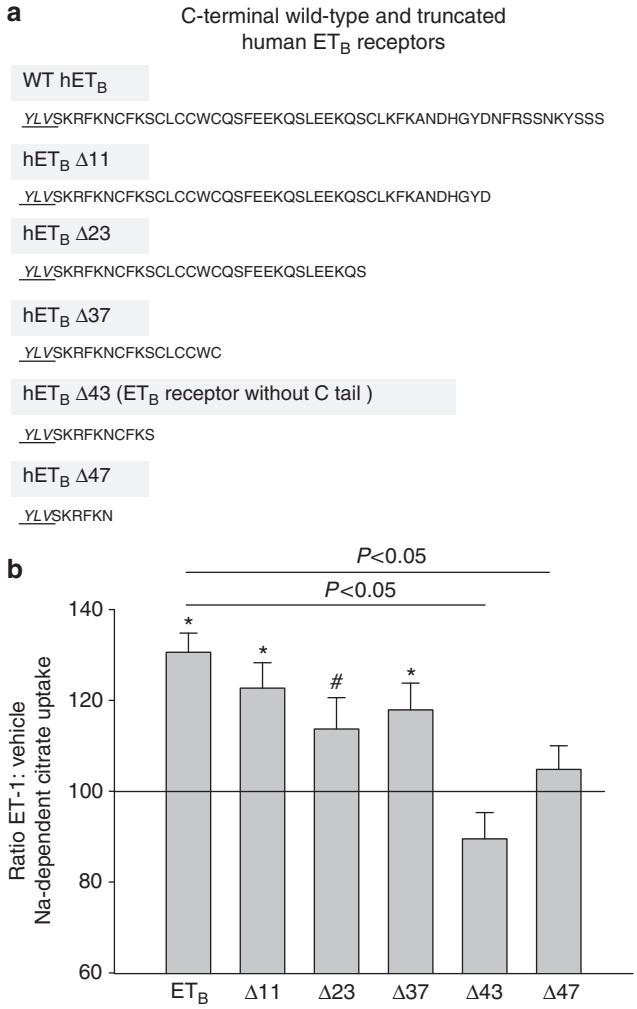

Figure 9 | Endothelin B (ET_B) C-terminal truncations alter ET-1 regulation of opossum kidney cell line Na-dependent dicarboxylate cotransporter-1 activity. (**a**) The wild-type (WT) human ET_B (hET_B) C-terminal sequence and five truncations are shown. The underlined and italized residues (*YLV*) represent the end of the last transmembrane domain. The Δ43 truncation is the construct used for the studies in Figure 8 labeled ET_B no tail. (**b**) Cells were transfected with oNaDC-1 and the WT or truncated receptor as indicated on the *x*-axis and [^{14}C]-citrate uptake was measured. $^*P<0.05$ versus vehicle; $^{\#}P=0.051$ versus vehicle. $n=27$ for ET_B; $n=9$ for Δ11, $n=18$ for Δ23, $n=21$ for Δ37 and Δ43, and $n=23$ for Δ47.

To identify the region of the ET_B C-terminal tail involved in ET-1 stimulation of oNaDC-1 activity, a series of C-terminal truncations were used. Figure 9a shows a schematic diagram of these truncations starting with the last three residues of the last TM domain (YLV). The specific truncations were made to (1) remove the most C-terminal five serine residues (Δ11), (2) remove Tyr430 (Δ23), (3) remove three additional serine residues (Δ37), (4) remove four cysteine residues, three of which are the known palmitoylation sites required for coupling with the G-protein signaling pathways (Δ43), and (5) remove the serine and cysteine residues closest to the seventh TM domain (Δ47).

As shown in Figure 9b, removing the terminal 11 amino acids (Δ11) does not significantly decrease the magnitude of ET-1 stimulation of oNaDC-1 activity. Removing the last 23 residues (Δ23) reduces the ET-1 effect by 50%, although the magnitude of ET-1 stimulation is still statistically significant and not different from the stimulation that occurs in the presence of the wild-type receptor. Removing another 14 resides (Δ37) has no additional effect on ET-1 stimulation of oNaDC-1 activity. However, removing the terminal 43 or 47 amino acid residues (Δ43 or Δ47) prevents ET-1 stimulation of oNaDC-1 activity. These latter truncations are consistent with the studies presented in Figure 8, showing that removal of the C-terminal tail (the Δ43 construct) prevents ET-1 stimulation of oNaDC-1 activity.

The ET_B C-terminal tail has relatively few identifiable consensus sequences that might suggest the specific key amino acid resides involved in ET-1 stimulation of oNaDC-1 activity. Between the Δ37 and Δ43 truncation sites, wherein ET-1 stimulation was blocked entirely, there are three palmitoylation sites, and between the Δ11 and Δ23 truncation sites, wherein 50% of the stimulatory effect was lost, is a SH2 binding domain. This latter domain contains a tyrosine residue, amino acid 430, one of only two tyrosine residues in the C-terminal tail, the other one being four residues from the C-terminal end of the protein and having no impact on ET-1 stimulation of oNaDC-1 activity when removed (Δ11).

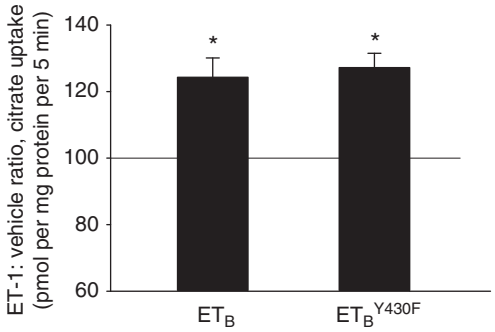

Figure 10 | Endothelin B (ET_B) C-terminal Tyr^{430} is not involved in ET-1 stimulation of opossum kidney cell line Na-dependent dicarboxylate cotransporter-1 (oNaDC-1) activity. Cells were cotransfected with oNaDC-1 and either the wild-type ET_B receptor or ET_B^{Y430F} mutant receptor, and [^{14}C]-citrate uptake was measured. *$P<0.01$ versus vehicle. $n=8$ for both groups.

To determine if Tyr^{430} has a role in ET-1 stimulation of oNaDC-1 activity, cells were transfected with oNaDC-1 and a mutant ET_B receptor in which Tyr^{430} was replaced with phenylalanine (ET_B^{Y430F}). As shown in Figure 10, removal of Tyr^{430} has no effect on ET-1 stimulation of [^{14}C]-citrate uptake.

DISCUSSION

In vivo proximal tubule apical membrane NaDC-1 activity increases in response to a dietary acid load and *in vitro* in response to media acidification.[11,13–15,27] This Na-dependent citrate transporter mediates most of citrate reabsorption in the nephron, and thus, transporter activity is a primary determinant of citrate excretion.[10] These studies establish that a functional ET_B receptor is required for acid stimulation of NaDC-1 activity both *in vitro* (OKP cells) and *in vivo* (comparing $ET_B^{+/+}$ with $ET_B^{-/-}$ mice).

Despite a dependence on the ET_B receptor for acid stimulation of NaDC-1 activity, as shown in Table 1, plasma [HCO_3] decreases to the same extend in $ET_B^{+/+}$ to $ET_B^{-/-}$ mice. In metabolic acidosis citrate excretion is reduced to minimize base loss in the face of base shortage. However, comparing the amount of bicarbonate generated from the metabolism of the retained citrate with the amount of bicarbonate generated by ammonium excretion, citrate metabolism accounts for $<10\%$ of the bicarbonate generated by these proximal tubule processes, and thus, is not a major determinant of systemic acid-base balance.

The reason proximal tubule citrate handling is of physiological and clinical interest in metabolic acidosis is that the hypocitraturia consequent of minimizing base excretion increases the risk for kidney stones. Of the urinary anions, citrate has the highest affinity for calcium. The calcium citrate complex is soluble, making the availability of citrate in the urine a key factor in determining the risk for kidney stone formation.[28] In response to an acid load or in conditions that decrease intracellular pH, such as chronic potassium deficiency, citrate reabsorption and metabolism increase, resulting in less citrate in the urine and an increased risk for kidney stone formation.[3,4,27,28]

To elucidate the acid-activated pathway that mediates stimulation of NaDC-1 activity and confirm that the findings are relevant to the *in vivo* setting, we use both *in vitro* (cell culture—media acidification) and *in vivo* (whole animal—acid feeding) models of an acid load. Even though the time course of these studies are different (cell culture studies conducted after hours of exposure to acid media and whole-animal studies conducted after days of acid feeding), several pieces of evidence suggest that the effects are mediated by the same acid-activated pathway: (1) the acid stimulatory effect on NHE3 and NaDC-1 activities in cell culture is absent in the presence of the specific ET_B blocker, BQ788 (*in vitro* model) and in BBMV harvested from $ET_B^{-/-}$ mice (*in vivo* model) (this paper and by Laghmani *et al.*[23] (2) The acid stimulatory effect on NHE3 and NaDC-1 activities in cell culture is absent after the transfection of a dominant-negative or siRNA Pyk2 construct (*in vitro* model) and in BBMV harvested from $Pyk2^{-/-}$ mice (*in vivo* model).[29–31] (3) Media acidification (*in vitro* model) leads to activation of c-Src and acid feeding (*in vivo* model) increases renal cortical c-Src kinase activity.[32,33] (4) Acid-induced phosphorylation of ATP citrate lyase is not blocked in the presence of the specific ET_B blocker, BQ788 (*in vitro* model), and is present in renal cortex harvested from $ET_B^{-/-}$ mice (*in vivo* model) (unpublished observations).

The OKP cell was used as the *in vitro* model, because in these cells media acidification has the same effect on other proximal tubule transporters as acid feeding does in the intact proximal tubule.[34–36] The OKP NaDC-1 cotransporter was cloned and shown to have biochemical characteristics consistent with rat, rabbit, and human NaDC-1.[19] These studies show that both media acidification and exposure to ET-1 in the presence of the ET_B receptor stimulate oNaDC-1 activity in both a dose- and time-dependent manner, with maximal uptake at a media pH of 6.8 that begins within 2 h of exposure to the acid media, and in the presence of 10^{-8} mol/l ET-1 that begins within 15 min exposure to the ET-1 (Figures 2 and 3). The likely reason for the difference in the time required for stimulation of NaDC-1 activity by acid (2 h) and ET-1 (15 min) is that acid stimulation of NaDC-1 activity requires the acid media to stimulate ET-1 synthesis, which then must be secreted and function in an autocrine manner to stimulate NaDC-1 activity through the ET_B receptor.[37]

For a similar reason, a possible explanation for the ET-1 effect being gone at 8 h but the acid effect remaining at 24 h is that the ET-1 added to the media has been degraded by 8 h, whereas in the acid studies, with the media still being acidic, the stimulation for ET-1 synthesis continues for 24 h (Figures 2 and 3).

In vitro (comparing media acidification-induced citrate uptake in the absence and presence of a specific ET_B receptor blocker) and *in vivo* (comparing acid feeding-induced citrate uptake in BBMVs made from $ET_B^{+/+}$ and $ET_B^{-/-}$ mice), acid

stimulation of NaDC-1 activity requires a functional ET_B receptor. We have previously shown in both OKP cells and kidney cortex that media acidification or acid feeding, respectively, increases ET-1 synthesis.[37,38] Taken together, these studies support the hypothesis that an acid load increases ET-1 synthesis, which then, in an autocrine or paracrine manner, signals through the ET_B receptor to mediate stimulation of NaDC-1 activity.[37]

The requirement for a functional ET_B receptor for acid and ET-1 stimulation of NaDC-1 activity parallels the requirement for a functional ET_B receptor for acid and ET-1 stimulation of NHE3 activity.[23,39,40] Increases in NHE3 activity enhance acid excretion, whereas increases in NaDC-1 activity decrease base loss. Thus, acid-induced adaptations in both NHE3 and NaDC-1 activities maximize the ability of the kidney to increase net acid excretion and minimize an effect on systemic acid–base balance.

In addition, identical is the observation that in the presence of the ET_A receptor, ET-1 fails to stimulate either NaDC-1 or NHE3 activity, and in fact, leads to an inhibition of transporter activity (this study)[20,40]. Although, additional studies from our laboratory showed that the ET_A C-terminal tail is necessary and sufficient for ET-1-induced inhibition of NHE3 activity, these studies show that the wild-type ET_A receptor (TM and C-terminal domains), not just the C-terminal tail is required for inhibition of NaDC-1.[40] Taken together, these observations raise the possibility that exogenous ET_A receptor expression sequesters a protein required for ET_B-mediated stimulation of oNaDC-1 and NHE3 activities and that the effective-binding site of this protein to the ET_A receptor may be different for oNaDC-1 and NHE3 regulation.

These studies also show that baseline (vehicle) NaDC-1 activity decreases in OKP cells transfected with the ET_A receptor. This observation is different than the effect on NHE3 activity, in which baseline activity is increased.[40] The reason for this difference is presently unknown.

Further studies were conducted to determine the region within the ET_B receptor that mediates ET-1 stimulation of NaDC-1 activity. Within the ET_B C-terminal tail 50% of the ET-1 effect is mediated by amino acid residues between residues 11 and 23 (counting backwards from the end of the C-terminus), although NaDC-1 activity is still significantly increased by ET-1 in the Δ23 construct and not different than in the presence of the wild-type ET_B receptor. There is a potential SH2-binding domain between residues 11 and 23 that contains a tyrosine residue at aa430. Although removal of these 12 amino acids reduces the ET-1 effect by 50%, replacing Tyr^{430} with phenylalanine has no effect on ET-1 stimulation of NaDC-1 activity, showing that Tyr^{430} is not required for ET-1 stimulation of NaDC-1 activity.

ET-1 stimulation is, however, totally abolished by removal of the amino acids between residues 37 and 43. The Δ43 and Δ47 constructs remove essentially all of the C-terminal tail. Our laboratory[40] and Okamoto *et al.*[41] have published receptor binding studies showing that in cells transfected with wild-type, chimeric, or mutated ET_B receptors, ET-1 binding is not different between the wild-type and various constructs. Although the lack of a stimulatory effect in the presence of the transfected, mutant receptor infers that these receptors are less active, it is also possible that the mutant receptors are dominant negative to the endogenous wild-type receptor. It is unlikely that such an effect would be attributable to limited agonist, but could be because of the cellular constituents that are required for receptor activity and are being sequestered by the transfected receptors. However, the conclusions of this study are unaffected whether the mutations create an inactive or a dominant-negative receptor, the key conclusion being that the receptor is no longer active and, thus, the physiological effect lost.

Okamoto *et al.*[41] showed that the palmitoylation sites (cysteine resides 402, 403, and 405) have a critical role in the coupling with G-proteins, regardless of the G-protein subtype, but do not have a role in receptor localization on the cell surface, ET-1 or ET-3 binding, or receptor internalization when bound to ligand. Single and combined mutations of the three cysteine residues showed that at least one of the three sites is required for G-protein signaling. In addition, C-terminal truncation removing C403 and C405, but leaving C402 showed that, in addition, to one palmitoylation site, activation of G_i (cAMP), but not with G_q (Ca^{+2}-dependent) pathways, also requires the C-terminal tail.

In these studies, removal of the terminal 23 or 37 residues (leaving all three palmitoylation sites intact) reduces the ET-1 effect, although the magnitude of the remaining stimulation is not statistically different than that seen with the wild-type receptor (Figure 9b). However, removing the three palmitoylation sites (Δ43 or Δ47 constructs) abolishes the ET-1 stimulatory effect, which taken together with the studies by Okamoto *et al.*, suggests that ET-1 stimulation of NaDC-1 activity is mediated by G-protein-coupled signaling. As we have shown that ET 1 stimulation of NaDC-1 activity does not involve cAMP, but requires Ca^{+2} for the full effect, these studies suggest that the ET-1 effect is mediated by a G_q pathway (unpublished results).

Thus, acid stimulation of the proximal tubule apical membrane transporters NHE3 and NaDC-1 requires a functional ET_B receptor in OKP cells and *in vivo*, and can be mimicked in OKP cells by exposure to ET-1. Similarly, the ET-1 effect leads to inhibition of both transport activities when ET_A receptors are exogenously expressed. However, necessary and sufficient regions of the ET_B receptor differ for stimulation of the two transporters. Although the ET_B C-terminal tail is necessary for maximal stimulation of both, it is only sufficient for ET-1 stimulation of NaDC-1 activity; the KXXXVPKXXXV sequence within the second intracellular loop is also required for stimulation of NHE3 activity.[40] Recent preliminary studies have identified another difference in the pathway that mediates ET-1 stimulation of NHE3 and oNaDC-1. ET-1 stimulation of NHE3, but not of oNaDC-1 activity is a Rho kinase- and stress fiber-dependent process.[31,42] Thus, it is possible that the ET_B receptor second

intracellular loop regulates signaling involved with Rho kinase activation and stress fiber formation, whereas the C-terminal tail is involved in another aspect of transporter stimulation that is common to both transporters, and sufficient for stimulation of NaDC-1 activity.

MATERIALS AND METHODS

Materials and supplies

All chemicals were obtained from Sigma Chemical (St Louis, MO, USA) except as follows: penicillin from Whittaker MA Bioproducts (Walkersville, MD, USA), culture media from GIBCO BRL (Grand Island, NY, USA); ET-1 from Peptides International (Louisville, KY, USA); [^{14}C]-citrate from Amersham (Arlington Heights, IL, USA).

Cell culture

OKP cells were passaged in high-glucose (450 mg/dl) Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). For experimentation, confluent cells were rendered quiescent by removal of serum, and media was changed to low glucose (100 mg/dl) Dulbecco's modified Eagle's media × 24 h.

NaDC-1 activity ([^{14}C]-citrate uptake)

Cells were plated in six-well culture plates, grown to 60–70% confluence, and transfected with 1 μg DNA using the pME18sf empty plasmid or the plasmid containing the human wild-type ET_A, wild-type ET_B, a chimeric, or a mutant receptor driven by the SRα promoter for over expressing the receptors and the pEGFP-C3 empty plasmid or plasmid containing oNaDC-1 driven by a SV40 promoter for over expressing oNaDC-1. All transfections were carried out using the Lipofectamin Plus kit (Invitrogen, Carlsbad, CA, USA) × 5 h.[43,44] Serum (10% fetal bovine serum) was then added to the wells × 19 h, after which the cells were 95–100% confluent. The cells were rendered quiescent × 24 h by the removal of the serum, and then exposed to one of the following experimental protocols. Cells were exposed to the same media at pH 7.4, 7.3, 7.2, 7.0, or 6.8, as noted for each experiment, or in the presence or absence of ET-1 or vehicle (0.0002% acetic acid). Media was acidified by the addition of HCl and then incubated in the tissue culture incubator for several hours before being added to the cells. We have shown that this approach leads to a consistent media pH.

[^{14}C]-citrate uptake was measured at pH 7.4 × 5 min in the presence (136 mM) or absence of NaCl (NaCl replaced with 136 mM choline Cl) using a solution containing, in addition to either NaCl or choline Cl, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$, 3 mM KCl, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.39 mM citric acid, and 0.5 μCi/ml [^{14}C]-citrate. The uptake reaction was stopped, and the remaining extracellular [^{14}C]-citrate removed by washing the cells three times with ice-cold 0.1 M $MgCl_2$ solution. After the last wash, the cells were lysed in 300 μl of 0.1 N NaOH and scrapped with a rubber policeman. A 10 μl aliquot was used to measure protein content by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA); the remaining 290 μl were put into 5 ml of scintillation fluid and [^{14}C]-citrate counted with a scintillation counter (UT Southwestern: Beckman LS3801; Yale: Packard Tri-Carb 2900TR). Uptakes are reported as pmoles citrate/mg protein/5 min.

Location of GFP-oNaDC-1 expression

OKP cells were grown on polyethylene terephtalate track-etched membranes and transiently transfected with GFP-oNaDC-1 and the ET_B receptor as described above. After transfection, cells were rendered quiescent by removing the serum × 24 h. For experimentation, cells were exposed to vehicle or 10^{-8} M ET-1 or control (pH 7.4) or acidic media (pH 6.8) × 35 min or 6 h, respectively. Cells were then washed with phosphate buffered-saline and inserts mounted on glass slides sealed with coverslips and nail polish. Confocal images were obtained using a laser scanning fluorescent microscope (LSM410; Zeiss, Thornwood, NY, USA).

Experimental animals

In vivo experiments were carried out in 4–5-month-old age- and sex-matched wild-type mice and mice in which the ET_B receptor was genetically disrupted by homologous recombination and rescued from aganglionic megacolon by expression of a *dopamine β-hygroxylase* promoter/ET_B receptor transgene.[22,24] Confirmation that the ET_B receptor was not expressed in the kidney in the rescued mice was obtained by polymerase chain reaction using a forward primer from exon 1 and a reverse primer from exon 3. As a control for loading, cyclophilin abundance was assayed and shown to be the same in wild-type and knock-out tissue.

Metabolic acidosis was induced by providing drinking water that contained 0.3 M NH_4Cl × 7 days; control mice received plain drinking water. After anesthetizing the mice with isoflurane by inhalation (Baxter, Deerfield, IL, USA), the kidneys were rapidly harvested and keep in 1 × phosphate-buffered saline on ice until the cortex was removed. Arterial blood was collected in anesthesized mice before harvesting the kidneys and blood gases measured using a Heska i-STAT portable clinical analyzer (Loveland, CO, USA).

BBMV isolation and measurement of NaDC-1 transport activity

NaDC-1 activity was assayed as [^{14}C]-citrate uptake in BBMV. Briefly, dissected kidney cortex from six kidneys was immersed in ice-cold buffer containing 300 mM mannitol, 5 mM ethylene glycol tetraacetic acid, 18 mM HEPES, and 0.1 mM phenylmethylsulfonyl fluoride titrated to pH 7.5 with Tris, and homogenized 5 s × 15 times with a Polytron homogenizer (Brinkmann Instruments, Westburg, NY, USA) followed by homogenization by 10 stokes in a Teflon-glass homogenizer at 4°C. BBMV were isolated using the Mg-ethylene glycol tetraacetic acid aggregation method.[4] The final BBMV fraction was loaded with intravesicular buffer (in mM) (200 Mannitol, 50 KCl, and 16 HEPES, titrated to pH 7.5 with Tris). A total of 45 μl extracellular buffer (in mM) (100 NaCl/100 Choline Cl, 50 KCl, and 16 HEPES, titrated to pH 7.5 with Tris) containing 0.1 mM [^{14}C]-citrate was added to 15 μl of BBMV (100–110 μg protein) at room temterature. After 5 s the reaction was stopped by addition of ice-cold stop solution (in mM) (135 NaCl, 10 Na_2 succinate, and 16 HEPES, titrated to pH 7.5 with Tris), the sample filtered through a 0.65 μm filter (Millipore Corp., Bedford, Massachusetts, USA), the filter put into 5 ml of scintillation fluid, and [^{14}C]-citrate counted using a Beckman scintillation counter (Packard Tri-Carb 2900TR, Downers Grove, IL, USA). [^{14}C]-citrate uptake was carried out in triplicate and meaned for a single value for the sample. Uptake is reported as pmoles citrate/mg protein/5 s.

Statistics

All multigroup statistical analyses were carried out using an analysis of variance and statistical significance determined by carrying out the appropriate *t*-test. Differences between means were considered significant at $P < 0.05$.

DISCLOSURE
All the authors declared no competing interests.

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